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Identification of resistance gene analogs in cotton (*Gossypium hirsutum* L.)

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Summary

Sequence analyses of numerous plant disease resistance genes have revealed the presence of conserved motifs common to this class of genes, namely a nucleotide binding site (NBS) and leucine rich repeat region. In this study, thirty-three resistance gene analogs (RGAs) were cloned and sequenced from cotton (*Gossypium hirsutum* L.) following PCR with degenerate primers designed from the conserved NBS motif of plant resistance (R) genes. Phylogenetic analysis of the predicted amino acid sequences grouped the RGAs into four distinct classes from which several subgroups were delineated based on nucleic acid sequences. Gene database searches with the consensus protein sequences of each of the four classes and respective subgroups of cotton RGAs revealed their conserved NBS domains and homology to RGAs and known resistance genes from a variety of plant genera. Given the complete lack of knowledge regarding molecular organization of R genes in cotton, the cloned RGAs described here may be useful as probes to map, characterize, and manipulate R genes of the cotton genome.

Introduction

Plants have evolved genetic mechanisms to withstand a diverse array of pathogens that include viruses, bacteria, fungi, nematodes, protozoa, and parasites (Staskawicz et al., 1995). The identification of many pathogen resistance (R) genes has increased understanding of how plants prevent or slow infection and disease progression (Hammond-Kosack & Jones, 1997). The plant R genes encode proteins that can recognize and bind products encoded by avirulence genes from the invading pathogens (Scofield et al., 1996). The recognition and binding events are thought to trigger a signaling cascade that induces various plant defense responses to impair pathogen growth and thereby limit disease (Dangl et al., 1996).

Many disease R genes have been cloned from a number of plant species (Baker et al., 1997). Although the R genes confer resistance to a wide range of pathogens, sequence analyses indicate significant similarities, particularly in the conservation of structural motifs. Numerous plant R genes encode proteins that contain leucine-rich repeat (LRR) domains (Kobe & Deisenhofer, 1994). These highly conserved domains provide pathogen recognition specificity by participating in protein-protein interactions and ligand binding (Kobe & Deisenhofer, 1994; Ellis et al., 2000). Only six amino acid changes in the LRR beta-strand/beta-turn motif were necessary to change from one rust resistance specificity (*P2* rust resistance gene) to another (*P* rust resistance gene) in flax (Dodds et al., 2001). Another conserved motif, a nucleotide-binding site (NBS), is present along with LRR motifs in many R genes (Bent, 1996). The NBS domains have been classified into two major groups based on the presence or absence of TIR (Toll/Interleukin-1 receptor) homologous sequences (Meyers et al., 1999; Pan et al., 2000). The NBS domain, together with either the leucine zipper or the TIR homologous domains in the NBS-LRR-containing R proteins, are thought to be in-

volved in activating the signaling events that lead to disease resistance (Hammond-Kosack & Jones, 1997).

It has been demonstrated that amplification of resistance gene analogs (RGAs) from plant species by polymerase chain reaction (PCR) was possible with the use of degenerate oligonucleotide primers based on conserved motifs from cloned plant R genes (Kanazin et al., 1996; Yu et al., 1996; Leister et al., 1996). Furthermore, restriction fragment length polymorphism (RFLP) loci identified by some of the RGAs were mapped in close proximity with known R genes, allowing identification of disease-resistance loci where R genes seem to be clustered (Kanazin et al., 1996; Yu et al., 1996; Leister et al., 1996). In this paper, we report the amplification of thirty-three RGAs from cotton (*Gossypium hirsutum* L.) by PCR using degenerate oligonucleotide primers based on conserved motifs of known R genes.

Materials and methods

Plant genomic DNA extraction

Plant genomic DNA was extracted from the leaves of root-knot nematode resistant cotton line M-249 (Shepherd et al., 1988, 1996) using the procedure described in Paterson et al. (1993). DNA samples were treated with 0.3 mg/mL RNase A (Sigma) at 37 °C for 1 hour. DNA concentration was estimated by a UV-VIS spectrophotometer.

Degenerate oligonucleotides and PCR strategy

Oligonucleotide primers used to amplify RGAs were based on four conserved amino acid motifs from the NBS of the *N* protein from tobacco, the *RPS2* protein from *Arabidopsis* and the *L6* protein from flax (Shen et al., 1998; Collins et al., 1998). The four conserved motifs are designated as the P-loop(kinase-1a), the kinase-2, the GLPLAL, and the MHD. Eight primers (P-loop1 to P-loop8) based on P-loop motifs and four primers (kinase-2D to kinase-2G) based on Kinase-2 motifs were orientated in the sense direction. Six primers (GLPL1 to GLPL6) based on GLPLAL motifs and four primers (MHD1 to MHD4) based on MHD motifs were orientated in the anti-sense direction. The specific sequences of these primer sets were described in Collins et al. (1998). The PCR experiments were conducted with a nested primer approach using the four primer sets. The first round of PCR was performed with all possible pairwise combinations of primers based on P-loop and MHD motifs. The PCR products were then pooled, diluted twenty fold, and used as templates for the second-round of PCR with all possible pairwise combinations of primers based on kinase-2 and GLPLAL motifs.

PCR amplifications and cloning of PCR products

PCR was performed according to Collins et al. (1998) with modifications. First round PCR was performed in 20 μL total volume containing 100 ng of genomic DNA, 0.2 mM each of the four dNTPs, 0.25 μm of each primer, 0.05 unit *Taq* DNA polymerase (Qiagen), 1X PCR buffer (Qiagen), and a final concentration of 5 mM $MgCl_2$. After an initial denaturing step at 94 °C for 1 min, forty cycles of PCR consisting of denaturation at 94 °C for 15s, annealing at 40 °C for 30s, and extension at 72 °C for 1 min were then performed in a DNA thermal cycler (Perkin-Elmer). The amplification was concluded with a final extension step at 72 °C for 7 min. The first round PCR products were pooled and diluted twenty fold, and 1 μL of the pooled and diluted samples were used as templates for the second round PCRs. The second round PCRs were carried out in the same volume and conditions as described above, except that the starting annealing temperature was 55 °C for 30s and was decreased in steps of 0.5 °C per cycle until 50 °C was reached. The reaction mixture was then subjected to another 25 cycles of PCR with annealing temperature at 50 °C for 30s. PCR products were analyzed by electrophoresis on 1% agarose gels in 1X TAE buffer containing 0.1 μg/mL ethidium bromide. PCR products of expected size were extracted, purified, and ligated to pGEM-T Easy vector (Promega) and transformed into competent cells (*E. coli* XL1-Blue) according to the supplier's protocol.

DNA sequencing and analysis

The recombinant pGEM-T plasmids were sequenced using the PRISM Ready Reaction DyeDeoxy Termination cycle sequencing kit with an ABI-310 Genetic Analyzer. Sequences with conserved NBS domains and homology to resistance genes or resistance gene analogs were identified using the NCBI BLAST network server and BLASTP 2.2.1 (Altschul et al., 1997). Alignment of translated sequences was done using Sequencher Version 4.0.5 software package. Phylogenetic analysis of amino acid sequences was performed via the Neighbor Joining Method (Saitou & Nei, 1987)

Figure 1. Character matrix of thirty-three *Gossypium hirsutum* Resistance Gene Analogs [GhRGA_(A-D)#] used to construct neighbor joining tree. Alignment of translated amino acid sequences was done with Sequencher 4.0.5. Primer sequences are excluded. Nucleotide and derived amino acid sequences for the cotton RGAs are available in the GenBank Database under accession numbers AF469073 (GhRGA_A), AF469074 (GhRGA_B), AF469075 – AF469089 (GhRGA_B1 – GhRGA_B15), AF469090 (GhRGA_C), AF469091 – AF469101 (GhRGA_C1 – GhRGA_C11), AF469102 (GhRGA_D), AF469103 – AF469105 (GhRGA_D1 – GhRGA_D3).

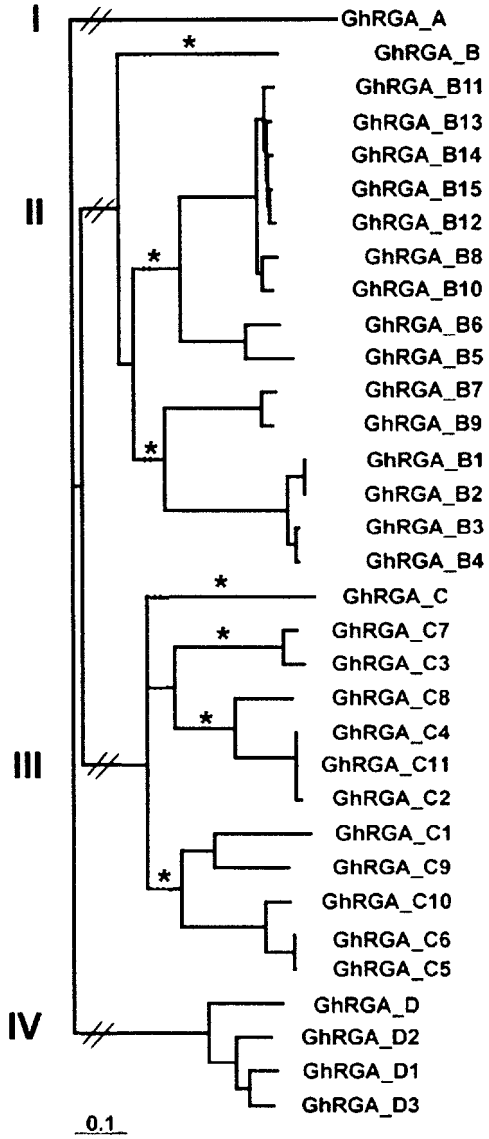

Figure 2. Neighbor joining tree of *Gossypium hirsutum* Resistance Gene Analogs [GhRGA_(A-D)#]. Branch lengths are proportional; scale for genetic distance is indicated as computed from the pairwise distance in PAUP. Major phylogenetic groups (I-IV) based on amino acid sequences. Subgroups(*), distinguishing very similar RGAs within a major group, were based on analysis of the nucleic acid sequences.

utilizing PAUP (Phylogenetic Analysis Using Parsimony, Version 4 for PC) based on mean character difference (distance).

Results and discussion

A nested PCR strategy was employed in an attempt to isolate RGAs from *G. hirsutum* genomic DNA using primer sets based on four conserved NBS motifs identified among plant disease R genes. The targeted four NBS domains included the P-loop (kinase-1a), kinase-2, GLPLAL, and MHD motifs. First-round PCR products generated by the P-loop and MHD primer combinations were used as templates for the second round PCR employing the kinase-2 and GLPL primer combinations. Since no introns are present between the kinase-2 and GLPL motifs in known plant disease R genes, only the expected size of approximately 250 bp amplified products from the second round PCR were cloned and sequenced. Seventy clones with continuous open reading frames were selected for analysis excluding primer sequences. BLASTP on the NCBI BLAST network server flagged conserved NBS domains in 33 of the clones, identifying them as potential RGAs. The predicted amino acid sequences of these 33 clones were aligned using Sequencher program (Figure 1). Such data represented the character matrix for derivation of a neighbor joining tree in PAUP (Figure 2). This analysis showed that the cotton RGAs can be separated into four distinct phylogenetic groups (I-IV). Several sub groups within these major RGA classes were delineated based on nucleic acid sequences (*, Figure 2). Interestingly, the lone member of Group I, clone GhRGA_A, was the only clone whose sequence contained the TIR (Toll/Interleukin-1 receptor) domain as identified by the BLAST search. NBS sequences identified in the other cotton RGAs included the kinase-2 (LIVLDD), kinase-3a, and GLPLAL motifs.

Given the close similarity of sequences within the designated phylogenetic groups, we derived consensus protein sequences representative of the major groups and respective subgroups in order to determine homology with known genes of the major databases. Table 1 shows the consensus sequences and most significant homologies obtained by BLASTP searches. All groups showed significant homology to resistance gene sequences from diverse plant species. Since in many cases the most significant probabilities were to RGAs (one EST) from other plant types, we included the most significant matches to known proteins where possible (Table 1). In this regard, Groups I, II-3, III-1, III-2, and IV showed homology to the major known R genes of plants, that is, tobacco mosaic virus resistance *N* protein (Whitham et al., 1994), flax

Table 1. Consensus amino acid sequences of phylogenetic groups of *Gossypium hirsutum* resistance gene analogs and homologous sequences obtained by BLASTP search at NCBI network server. Group designations are from neighbor joining tree (Figure 2). Probability values from homology searches are considered significant at <0.05 (Karlin & Altschul, 1990)

Consensus amino acid sequence	Homologous GenBank accessions	% Identity	Probability
Group I			
T E I V K Q K N D K G Y K V F L V F Y D V D P S D L	Tobacco N gene homolog,	48	7e-13
R K Q K G K V E E A F A E H E K R Y D E D I L Q R W	*Solanum tuberosum* (CAC82812.1);		
R N A L I Q V A N I K G W H L N R R L C W	Flax rust resistance protein, *Linum usitatissimum* (AAD25969.1)	39	6e-11
Group II			
Subgroup 1			
W H E N R A R W I E L R D I L G S M D Y L C G S K I	EST: NBS-LRR resistance protein,	47	1e-13
I V T T R S L K V A F I M S S I H P Y E L K G L P F	*Oryza sativa* (BAB03441. 1);		
E D C L T L F I K W A F N N E D E R Q Y P N L M R I	RGA: NBS-LRR resistance protein,	42	2e-13
G K E I V Q K C K	*Theobroma cacao* (AAL01021.1)		
Subgroup 2			
L L V L D D V W N E K Y V D W E E L R S P F C F G A	Unknown protein,	50	2e-21
K N S K I V V T T R N E S V A S I M R T V P T Y H L	*Cajanus cajan* (AAF36346.1);		
N I L S D E D C W G L F A K H A F V D T S P S M H P	RGA: NBS-LRR resistance protein,	51	5e-20
N L I A T S E A M V K R C R	*Theobroma cacao* (AAL00976.1)		
Subgroup 3			
W N E N Y H N W T I L Q S P F L T K T K G S K V I V	RGA: NBS-LRR resistance protein,	54	2e-23
T T R N H G V S S T M G A F H A H X L E V L S D D A	*Theobroma cacao* (AAL00991.1);		
C L S I F A Q H A L G A R D F G G H P N L K E V A K	*I2*, fusarium resistance protein,	44	7e-15
K I V R K C N G L P L A F S S	*Lycopersicon esculentum* (AAD27815.1)		
Group III			
Subgroup 1			
W E S I D L E A V G I P N P S S E N G S A T I L A T	RGA: Disease resistance-like protein,	43	2e-12
R N L E V C N N M R F I N M I E V G T L S N E E A W	*Brassica rapa* (AAK18300.1);		
K L F C E Q V G R V V N I P G I L P F A R V I A E R	*RPS2* like protein,	40	5e-11
C G	*Arabidopsis thaliana* (NP_193197.1)		
Subgroup 2			
T V A G R C L L L L D D V W E K V S X E X V G I P E	RGA: NBS-LRR resistance protein,	54	3e-22
S S N X S K L V L T T R S L D V C R H V G C N R V I	*Theobroma cacao* (AAL01014.1);		
Q I K P L X E X E A W N L F L E I V G G N I L N I P	Disease resistance *RPS2*-like protein,	43	9e-12
G L E P V A K S I T K H C A	*Arabidopsis thaliana* (CAB78503.1)		
Subgroup 3			
L V I L L V L D D V W S E V S L E E I G I P E P S S	RGA: NBS-LRR resistance protein,	40	4e-13
S N G Y K L V L T T R V E Q V C K S M G C K V I K V	Theobroma cacao (AAL01028.1);		
K P L S E E E A L I L S L S E V G P N I V Q N Q T I	RGA: resistance protein,	40	4e-9
M P T L K L V V K E C A	*Elaeis guineensis* (AAF24311.1)		
Subgroup 4			
W S S F P L E D I G I I E P T X D N G C K V V L T T	RGA: NBS-LRR resistance protein,	64	1e-25
R S E E V I R S M G C K K V Q V A C L S M H E A M N	*Theobroma cacao* (AAL01005.1);		
L F L S K V V Q D I S E N P T L K S S M R L A V G E	RGA: disease resistance-like protein,	46	2e-13
C E G L P L A L S S	Brassica napus (AAG40132.1)		
Group IV			
L L L L D D V D N L Q H L K C L V G X R D W F G L G	RGA: resistance protein analog,	51	3e-19
X R I I V T T R D E H L L R S Y R V D G V Y K P T T	Phaseolus vulgaris (AAF81616.1);		
X K X N D A L H L F N L K A F G C E T X P K E D F I	Downey mildew resistance protein *RPP5*,	46	6e-14
E L A K H I V G Y A G	Arabidopsis thaliana (AAF08790.1)		

rust resistance protein (Lawrence et al., 1995), tomato I_2 *Fusarium* resistance protein (Ori et al., 1997; Simons et al., 1998), *Pseudomonas* resistance protein *RPS2* (Bent et al., 1994; Mindrinos et al., 1994) and downey mildew resistance protein *RPP5* in *Arabidopsis* (Parker et al., 1997). Group III-4 consensus sequence showed the overall most significant match (64% identity, 1e-25 probability) to an RGA from cocoa (*Theobroma cacao*) (Table 1).

The use of oligonucleotide primers based on conserved domains of known plant disease R genes in PCR has facilitated many groups to amplify RGAs with conserved motifs similar to known resistance genes (Kanazin et al.,1996; Leister et al., 1996; Yu et al., 1996; Collins et al., 1998; Seah et al., 1998; Shen et al., 1998; Joyeux et al., 1999; Rivkin et al., 1999; Deng et al., 2000; Di Gaspero & Cipriani, 2002). Many of these RGAs were observed to cluster within R gene loci. The number of genes found within a cluster can be as few as two at the *Cf*-2 locus in tomato (Dixon et al., 1996) to as many as 24 at the *Dm*3 locus in lettuce (Meyers et al., 1998). Many of these different R gene loci were shown to link tightly with other R gene loci within a small genetic distance (Meyers et al., 1999). Speulman et al. (1998) demonstrated that the map positions of RGAs correspond to 21 disease resistance loci in *Arabidopsis*. Shen et al. (1998) mapped two of the four families of RGAs identified in lettuce in close proximity to known disease-resistance clusters. Since RGAs cluster in close proximity to disease-resistance loci, a potential use of the cotton RGAs could be as probes to identify polymorphisms in resistance genes from diverse cotton germplasm. RGA-derived RFLP markers that cosegregate with the pathogen-resistance phenotype, when converted into a PCR-based marker, could provide an assay for selection of resistant progeny in breeding programs. Recently, an RGA-derived PCR assay has demonstrated the ability to detect the presence of the introgressed rust resistance genes across a diverse wheat background (Seah et al., 2001). Deng et al. (2000) were able to utilize RGA-based CAPS (cleaved amplified polymorphic sequence) markers to associate closely with two important resistance genes, the citrus tristeza virus resistance gene (*Ctv*) and the citrus nematode resistance gene (*Tyr 1*) in *Poncirus trifoliata*. A number of restriction enzyme and RGA probe combinations should enhance the probability in detecting more polymorphic CAPS or RFLP markers. Furthermore, more RGAs can be detected by extensive design of degenerate primers based on different sets of NBS R gene classes. Given the paucity of knowledge regarding molecular organization of R genes in cotton, the cloned RGAs described here may be useful as probes to map, characterize, and manipulate R genes of the cotton genome. Of particular interest to us will be the use of the cotton RGAs as RFLP probes on near-isogenic breeding lines of cotton where unidentified genes for resistance to root-knot nematode cosegregate with *Fusarium* wilt resistance (Shepherd, 1974).

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